

Issue of inventiveness*State of the art*

The state of the art is the method to obtain full-length APP recombinant protein based on the isolation of APP-cDNA clones from the cDNA library using the synthesized oligonucleotide probes which is very time-consuming and requires highly skilled personnel to perform.

Contribution over the art

The present application offers a new method to overcome the above mentioned technical problem. What is inventive and what a person of ordinary skill in the art would not do is to conceptualize a different approach for obtaining different powerful expression constructs most easily and rapidly in order to get full-length recombinant APP protein. This is not just a matter of using prior known means, well known compounds, well known devices, or well known techniques of the field of molecular biology as described by Games, Kang, Powell, Buck, Innis, Brook and Essamani. As a matter of fact they have not indicated the way to resolve the technical problems to obtain full-length APP recombinant protein in a fast and easy way.

Thus, what is inventive and what a person of ordinary skill in the art would not do is the present invention: The new procedure to **get the APP constructs using the easiest and fastest method via the commercially available expression plasmid vectors in order to get full-**

length recombinant APP protein in different expression systems. This procedure consists of:

1) Performing the RT-PCR reactions from the total RNA in order to get the PCR products

corresponding to the CDS (~2.3 kb) of the APP gene that encodes full-length APP protein, APP - cDNA (Claim 1); and 2) ligating the APP -cDNA PCR products to the selected commercially available expression plasmids vectors in order to get the APP constructs capable of producing

full-length recombinant APP protein in different expression systems, such as baculovirus and bacterial expression systems (Claims 2 and 3).

What is inventive and what a person of ordinary skill in the art would not do is to figure out the specific, easiest and fastest procedure to focus on, in order to get the APP constructs
5 capable of producing rapid, high yield and stable full-length APP recombinant protein.

Once this applicant decided that the work should be done via RT-PCR reactions from the total RNA, what is inventive and what a person of ordinary skill in the art would not do is to
10 identify the RT-PCR conditions in order to get directly and specifically the PCR products corresponding to the CDS (~2.3 kb) of the APP gene that encodes full-length APP protein: APP -cDNA.

Once the APP-cDNA is obtained, what is inventive and what a person of ordinary skill in the art would not do is to identify the approach in order to get the APP constructs easily and rapidly. The point is to determine what steps to take, then determine which techniques are appropriate to perform the needed tasks.

CLAIM 1: OBTAINING THE HUMAN APP RT-PCR PRODUCTS

Concerning Claim 1, Office Action (pages 9 and 10) states that:

20 “Routine optimization is not considered inventive and no evidence has been presented that the selection of the claimed RT and PCR reaction conditions was other than routine or that the results should be considered unexpected in any way as compared to the closest prior art.

Therefore, the person of ordinary skill in the art, interested in obtaining a human β A precursor protein gene product, would have been motivated to perform the RT-PCR method taught by Games using any primer set derived from the known human β A precursor protein gene sequence, as suggested by Powell, Kang, and Buck, under optimized reaction conditions, as
25 suggested by Innis, thus resulting in the instantly claimed method.”

Not a task for a person of ordinary skill in the art to perform

From the identification of the sequence of APP gene as described by Kang et al. (GenBank Accession No. Y00264), this applicant has conceptualized a new approach for obtaining different powerful expression constructs most easily and rapidly in order to get full length APP recombinant protein. The following explanation will demonstrate that this applicant's claim 1 is over Games, Powell, Bruce, and Innis.

Reverse Transcription reaction in claim 1

It must be clarified herein that, contrary to what is mentioned in the Office Action about the one-step RT-PCR reactions performed by Games (Office Action, p. 6), Games actually performed two-step RT-PCR reactions using oligo-dT primers for RT reaction and two primers for PCR reaction (see Games et al. in U.S. Patent 6,717,03 and the RT-PCR reactions described by Wang et al., Proc. Natl. Acad. Sci. U.S.A. 86:9717-9721, 1989).

Over Games who uses oligo-dT primers which result in cDNA NON-SPECIFIC, this applicant uses the **specific primer** (SEQ ID NO: 1) to perform the reverse transcription reaction (RT) which recognizes the SPECIFIC APP-mRNA resulting in APP-cDNA.

The state of the art is reflected in Powell's statement (quoted on page 6, Office Action) – "One of the advantages of using specific primers close to the site of modification is that cDNA synthesis and subsequent amplification may be performed on less than intact preparations of total RNA" – which indicates the vague state of knowledge at the time. **Over Powell, this application indicates advancement toward a more precise piece of knowledge by demonstrating the main advantage of using specific primers for RT reaction (SEQ ID NO: 1): The main advantage is to get the SPECIFIC cDNA which results in obtaining the expected PCR products specifically and directly via PCR amplification.**

Not a task for a person of ordinary skill in the art to perform

Polymerase Chain Reaction in claim 1

In response to the Office Action, clarifications regarding Polymerase Chain Reaction (PCR) amplification need to be first brought forth.

5 Concerning PCR amplification, the selection of the specific primers is based on the sequence of the target gene. Contrary to what is taught by Buck (Office Action p. 6, 7) – “...This clearly shows that every primer would have a reasonable expectation of success” (p. 7) – Buck’s statement is inaccurate. The fact is that not all selected primers have the same probability of reasonable success. As a matter of fact, some of the selected primers have no success at all in
10 PCR amplification! In general, for a given selected primer, besides the issue of selecting the specific primers, the success in getting the expected PCR product also depends on the PCR reaction conditions, such as time and/or temperature of the denaturation, and annealing and extension steps. **Contrary to Buck’s teaching, this applicant selected the specific primers (SEQ ID NOS: 2 and 3), and optimized the conditions for PCR amplification by taking into
15 account the specific properties (base composition, length and concentration) of the target gene and primer sequences.**

In PCR reaction, Innis teaches that “the number of cycles should be optimized based on the specific properties (base composition, length, concentration) of the target and primer sequences.” (Office Action p. 7). **Over Innis, this applicant teaches the number of cycles for
20 PCR amplification should be determined by taking into account the objective of the research work.** If the objective is to get a high quantity of PCR products, the number of cycles of the PCR amplification would be 30 to 40 cycles. However, for research work using a quantitative method based on PCR amplification, the number of cycles of the PCR amplification

would be 20 to 25 cycles in order to ensure quantitative measurements during the linear phase of the PCR amplification.

Contribution over the art related to claim 1

This applicant's contribution over the art is in:

5 -Selecting the specific primers for RT reaction (SEQ ID NO: 1) in order to get the specific APP-cDNA containing the CDS of the APP gene;

 -Selecting the specific primers (SEQ ID NOS: 2 and 3), and optimizing the PCR reaction; conditions by taking into account the specific properties (base composition, length and concentration) of the target gene and primer sequences in order to get the PCR products
10 corresponding to the CDS of the APP gene;

 -Determining the number of 35 cycles for PCR amplification in order to get high quantity of PCR products;

The above mentioned methods thus lead to the contribution of the art demonstrated through the actual result of clear and obvious specificity as shown by only one band, with high

15 quantity of 2.3 kb of the PCR product corresponding to the CDS of the APP gene obtained by electrophoresis analysis on a 20 g/L agarose gel (see attached copy of Figure 1 of the

application). In the field of molecular biology, obtaining only one band, with high quantity of the PCR product is the real proof of the applicant's contribution over the art in terms of the specificity of the selected primers (SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3) and the

20 efficacy of the optimized conditions for RT-PCR reactions as described in claim 1. Otherwise, if there is a lack of specificity from the selected RT-PCR primers stated in claim 1, the amplification of the CDS of the APP gene cannot take place; in this case, there would be no band of 2.3 kb of the PCR products. A best situation due to lack of specificity of the selected RT-PCR

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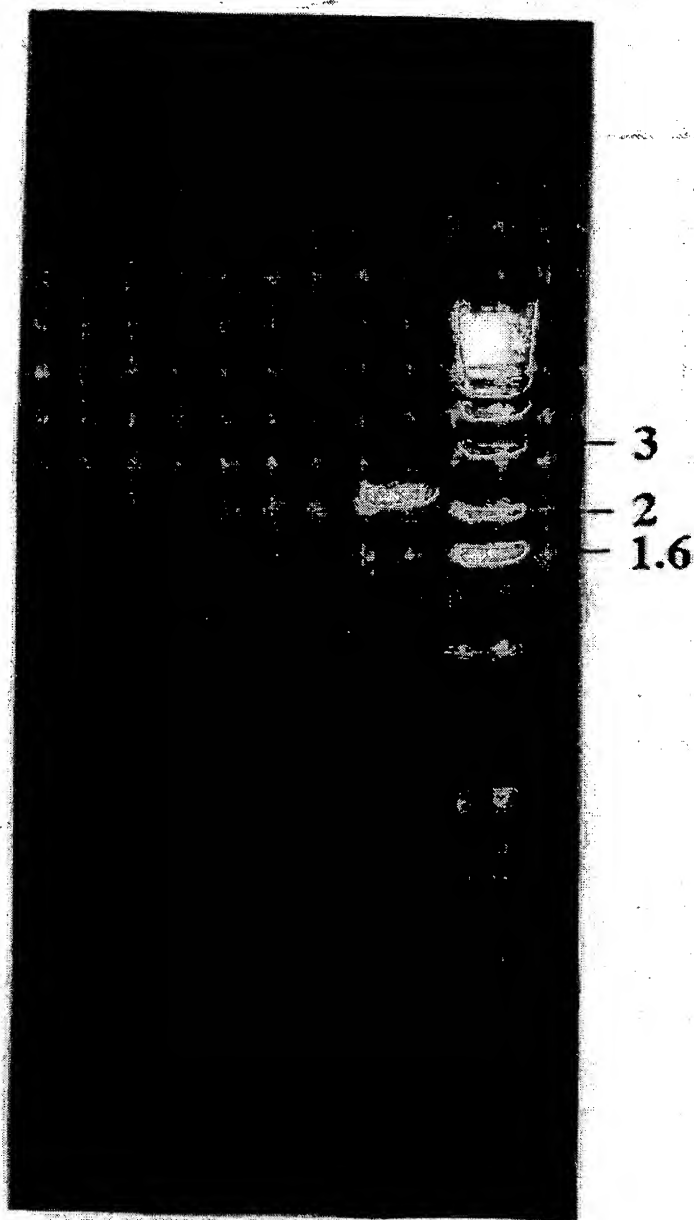


Fig. 1

primers would not result in the amplification of the CDS of the APP gene alone but also the amplification of other different genes; in this case, other different bands of different sizes would be present, besides the band of 2.3 kb corresponding to the CDS of the APP gene product.

5 CLAIMS 2 and 3: OBTAINING THE EXPRESSION CONSTRUCTS OF THE HUMAN APP
GENE

Concerning claim 2, Office Action (p. 10-13) states that:

10 “Essalmani teaches construction of vectors (pAcCL29) containing the human amyloid precursor protein gene (APP) from cDNA containing the APP695 gene and expression of the gene and cotransfection of insect cells with the plasmid and linear viral DNA to obtain recombinant baculoviruses for reinfection of insect cells for expression of the gene (p. 157, column 2, lines 16-27).

15 Essalmani does not teach transfer of the APP gene from a pCR®II vector to a pFastBac vector followed by transformation into INVαF’ and DH10Bac cells, colony screening and sequencing.” (p. 10, 11).

20 “Essalmani taught expression constructs of the human APP gene using a variety of plasmid vector construct and coinfection of insect cells to produce baculovirus vectors (p. 389, column 2, lines 16-27, see above), but did not specify a preferred baculovirus expression vector system.” (p. 13).

Not a task for a person of ordinary skill in the art to perform

The following explanation will demonstrate that this application’s claim 2 is over
Essalmani.

25 A. Essalmani does not teach what this **applicant has done in terms of conceiving and developing the method how to perform the ligation of the PCR product, cDNA of the CDS of the APP gene (APP-cDNA) into the pCRII plasmid vector (SEQ ID NO. 4) in order to obtain vector (1) (SEQ ID NO. 4 / APP₇₅₁-cDNA) and vector (2) (SEQ ID NO. 4 / APP₇₇₀-cDNA).** In light of this procedure, a point of clarification must be noted here: At the time of this invention, the ligation reaction
30 using ligase enzyme was actually needed for the construction of the vectors (1) and

(2) from the pCRII TOPO vector of the Invitrogen TOPO TA cloning manual kit – which is different from the new version of 2001 mentioned in the Office Action (p. 12, 13) concerning the use of the pCRII TOPO not requiring ligase.

B. What Essalmani does not teach – **the step for the construction of the vector (1)**

5 **(SEQ ID NO. 4 / APP₇₅₁-cDNA) and vector (2) (SEQ ID NO. 4 / APP₇₇₀-cDNA) –**
is what this applicant has developed; and this is crucial for the construction of the expression plasmids for APP protein using the pFastBac HTb vector (Baculovirus Expression System). Indeed, the pFastBac HTb vector has Xba I and Hind III restriction sites in their multiple cloning sites. The Xba I and Hind III restriction sites
10 are present in the pCRII vector. However, these restriction sites are absent in the cDNA of the CDS of the APP gene (APP-cDNA). Therefore, the issue is: How does one create the Xba I and Hind III restriction sites on the cDNA of the CDS of the APP gene in order to ligate to the expression vectors pFastBacTM HTb? The state of the art does not address this issue as reflected in Sambrook's statement (Office Action
15 p. 13) which indicates a vague state of knowledge at the time.

C. It is this applicant who resolved the above mentioned issue. This applicant performed the transfer of the APP-cDNA to the pCRII vector in which the Xba I and Hind III restriction sites are already present. Then, the applicant obtained the APP -cDNA with the Xba I and Hind III restriction sites by digestion of vector (1) and vector (2)
20 with Xba I and Hind III restriction enzymes. Then, this applicant took the APP-cDNA with the obtained Xba I and Hind III restriction sites to ligate to the expression vector pFastBac HTb (Bac-to-Bac Expression System), which thus results in vector (3) (SEQ ID NO. 5 / APP₇₅₁-cDNA) and vector (4) (SEQ ID NO. 5 / APP₇₇₀-cDNA)

to use for the expression of recombinant APP protein in insect cells. **Over the art is this applicant's creation of the Xba I and Hind III restriction sites on the cDNA of the CDS of the APP gene (APP-cDNA) by selecting the commercially available pCRII vector that has Xba I and Hind III. What is new is the fact that the**

5 **applicant has identified this method which is the easiest and fastest way to get such a PCR product.** The state of the art has been the creation of the Xba I and Hind III I restriction sites on the cDNA of the CDS of the APP gene obtained by performing the PCR amplification of the CDS of the APP gene using the specific primers supplemented with the nucleotides corresponding to those of the Xba I and

10 Hind III I restriction sites; this procedure is laborious because it requires the selection of such primers and the optimization of the PCR amplification in order to get such PCR product.

Next, to perform the construction of vector (7) (SEQ ID NO. 6 / APP₇₅₁-cDNA) and vector (8) (SEQ ID NO. 6 / APP₇₇₀-cDNA) of the Bac-N-Bac Baculovirus Expression

15 System to use for the expression of recombinant APP protein in insect cells, the **applicant isolated the APP-cDNA with the Nco I and Hind III restriction sites by digestion of vectors (3) and (4) with Nco I and Hind III restriction enzymes in order to ligate to the expression vector pBlue Bac His 2A.**

D. Essalmani does not teach the use of INVαF' and DH10 Bac cells, colony screening

20 and sequencing (Office Action, p. 11). **Over Essalmani**, the applicant deemed that INVαF' cells are ideal for propagating plasmids and cDNA library construction and used them for checking the success of the construction of the vectors (1), (2), (3), (4), (7), (8) (9) and (10). The applicant used the DH10 Bac cells containing a parent

bacmid DNA with a lac z-mini-att Tn fusion as a source of bacmid needed for transfection of insect cells in the next steps. The success of the transposition between the insert APP-cDNA from the vector (3) (SEQ ID NO. 5 / APP₇₅₁-cDNA) and vector (4) (SEQ ID NO. 5 / APP₇₇₀-cDNA), and the bacmid is visualized by the presence of the white bacterial colonies due to the expression cassette that disrupts the lac z gene. The transfection of the insect cells is performed with the recombinant bacmids DNA (5) and (6).

E. Essalmani teaches expression constructs of the human APP gene using a variety of different vectors, including baculovirus vectors, but does not specify a preferred baculovirus expression vector system (Office Action, p. 13). **Over Essalmani**, this applicant selected specially the pFastBac and pBlue Bac His 2A vector to obtain a rapid and stable expression of recombinant APP protein, capable of being purified using simple, one-step affinity-based methods.

Concerning claim 3, Office Action (p. 15) states that:

“Essalmani teaches construction of plasmid vectors (pAcCL 29) containing the human amyloid precursor protein gene (APP) from cDNA containing the APP₆₉₅ gene and expression of the gene and cotransfection of insect cells with the plasmid and linear viral DNA to obtain recombinant baculoviruses for reinfection of insect cells for expression of the gene (p. 157, column 2, lines 16-27).

Essalmani does not teach an expression plasmid system using the pET-28a(+) bacterial expression vector. Essalmani also does not teach transfer the APP gene from the pFastBac vector followed by transformation into INVαF' cells and colony screening.” (p. 15).

Not a task for a person of ordinary skill in the art to perform

Over Essalmani, this applicant selected the pET-28a(+) vector in order to get a rapid and stable expression of recombinant APP protein, capable of being purified using simple, one-step affinity-based methods.

5 **Over Essalmani**, in order to perform the construction of vector (9) and vector (10) (pET-28a(+) / APP-cDNA) (Prokaryotic Expression System) to use for the expression of recombinant APP protein in E. Coli cells, this applicant isolated the APP-cDNA by digestion of vectors (3) and (4) with Sal I and Hind III restriction enzymes in order to ligate to the expression vector pET-28a(+).

10 **Contribution over the art related to claims 2 and 3**

The applicant's contribution over the art is in:

-Developing the easiest and fastest way to get the APP constructs by ligating the obtained APP-cDNA products to the selected most powerful expression vectors commercially available;

15 -Developing the easiest and fastest way for creating the Xba I and Hind III restriction sites on the obtained APP-cDNA products by ligating them to the PCR II vector commercially available, which result in vectors (1) and (2);

20 -Developing the easiest and fastest way to get the APP constructs (3) and (4) from the obtained vectors (1) and (2) by digestion of these vectors (1) and (2) with Xba I and Hind III restriction enzymes and by ligation of the resulting fragment to the expression plasmid vector pFastBac HTb which resulted in vectors (3) and (4) respectively;

-Developing the easiest and fastest way to get the APP constructs (7) and (8) from the obtained vectors (3) and (4) by digestion of these vectors (3) and (4) with Nco I and Hind III

restriction enzymes and by ligation of the resulting fragment to the expression plasmid vectors pBlue Bac His 2A which resulted in vectors (7) and (8) respectively;

-Developing the easiest and fastest way to get the APP constructs (9) and (10) from the obtained vectors (3) and (4) by digestion of the vectors (3) and (4) with Sal I and Hind III

5 restriction enzymes and by ligation of the resulting fragment to the expression plasmid vectors pET-28(+) which resulted in vectors (9) and (10) respectively;

-Utilizing the INVα F' cells for checking the success of the construction of the vectors (1), (2), (3), (4), (7), (8), (9) and (10).

10 Conclusion

This application is over the art because the inventor conceived and developed a new approach using 1) RT-PCR reactions in order to get the PCR products corresponding to the CDS of the APP gene that encodes full-length APP protein, and 2) powerful commercially available expression vectors to get the different APP constructs most easily and rapidly in order to rapidly
15 produce a high yield of full-length recombinant APP protein, capable of being purified via a simple, one-step affinity-based purification process. No one before has developed the easiest and fastest method of obtaining the APP constructs via RT-PCR reactions and via the use of powerful expression vectors commercially available.